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TRANSLATOR'S CERTIFICATE

I, Bettina Cornelia Price, residing at 57, Beech Lane, Earley, Reading, RG6 5QA, Great Britain, declare:

That I am thoroughly conversant with the German and English languages;

That I have carefully made the attached translation from the original document written in the German language;

That the attached translation is a true and correct English version of such original, to the best of my knowledge and belief;

I further declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the patent or trademark.

Dated: 12/29/2001

(Bettina Price)

GK-OEH-119/500814.20020

STABLE NEAR-INFRARED (NIR) MARKER DYES BASED ON BENZOPYRYLIUM-POLYMETHINES

The invention is directed to so-called laser-compatible NIR marker dyes based on polymethines for use in optical, in particular, fluorescence optical determination and detection methods. Typical applications of the process are based on the reaction of dye marked antigens, antibodies, ligands or DNA segments with the respective complementary species.

Possible uses exist, for example, in the fields of medicine, pharmaceutics and in the areas of life science, materials science, in environmental monitoring and in the detection of organic and inorganic micro-samples occurring naturally and in technological contexts, but they are not limited to the aforementioned fields.

The usability of polymethines as NIR markers has been known of for a long time; they distinguish themselves by their strong absorption maxima which can easily be transposed into the NIR range (Fabian, J.; Nakazumi, H.; Matsuoka, M.: Chem. Rev. 1992, 92, 1197). With a suitable substituent pattern and pi-electron system and at a sufficient quantum yield they also fluoresce in the red and near infrared (NIR) range. Correspondingly, these compounds are widely used in different technological fields: as sensitizers in AgX materials, as laser dyes, as quantum counters, as indicator dyes in sensor technology, as light absorbers in writable CDs and last but not least as biomarkers ("Near-Infrared Dyes for High Technology Application", published by Daehne, S.; Resch-Genger, U.; Wolfbeis, O.-S., Kluwer, Academic Publishers – Dordrecht/Boston/London – 1998).

The number of polymethines used as biomarkers is limited. So far, only the trimethine Cy3 derived from astraphloxine (DE 410 487), the vinylogous pentamethine Cy5 and the doubly vinylogous heptamethine Cy7 with absorption maxima at approximately 550nm, approximately 650nm and approximately 750nm have so far found wide commercial application in this manner (US PS 5 627 027).

Also available are the polysulfonated trimethine Cy3.5 derived from the commercial hepatmethine "Indocynaninegreen" or "Cardio Green" and the pentamethine Cy5.5 (US PS 5 569 766). Heptamethines with aliphatic bridges in the polymethine chain have been developed by Patonay (US PS 5 800 995). All commercial biomarkers are characterized by terminal heteroaromatics derived from indene or heteroindene (Fischer's base). If methylsubstituted cycloimmonium salts are used as terminal polymethine building blocks, it is necessary to arrange at least five sequential sp^2 hybridized carbon atoms (pentamethines) between the heterocycles to generate absorption maxima at the boundary to the NIR range.

The NIR polymethines used in technology as biomarkers have the distinct disadvantage that lengthening the polymethine chain increases the opportunities for nucleophilic or electrophilic attack on the chain, in consequence of which the pi-system is destroyed. Further disadvantages of these marker dyes consist in their insufficient photostability and stability in storage, complicated synthesis and purification stages, low absorption coefficients/low fluorescent quantum yields as well as undesired changes of their optical properties in the presence of or after bonding with proteins or nucleic acid oligomeres. For example, a reduction of the fluorescent quantum yield of Cy5 has been described for the covalent bonding with different albumins (Oswald, B.; Patsenker, L.; Duschl, J.; Szmacinski, H.; Wolfbeis, O.S.; Terpeschnig, E.; Bioconjugate Chem. 1999, 10, 925-931).

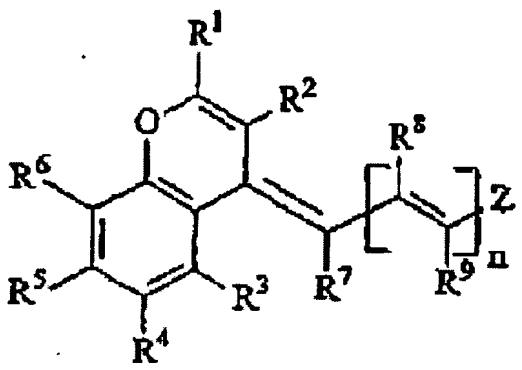
The use of pyrylium and benzopyrylium heterocycles or the corresponding mesomeric chromenes as terminal end groups in marker dyes in biologically relevant systems is so far not known in the art. This is due to the extreme sensitivity to hydrolysis of these pi-deficient aromatics, especially in an aqueous basic environment (H. Lietzt, G. Haucke, P. Czerney, B. John, J. Prakt. Chem., 1996, 338, 725-730).

Telfer et al. (US Patent 5 262 549) describe symmetrical trimethines based on 2-alkyl substituted benzopyrylium salts for the use as NIR absorbers in polimeric media with a reduced tendency towards aggregation in these media.

The aim of the invention was to create NIR marker dyes based on polymethine which have a high degree of photostability and stability in storage as

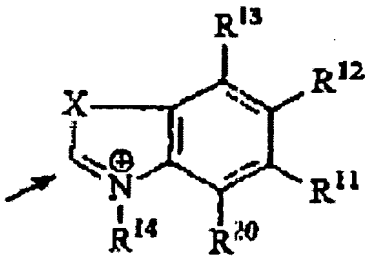
well as a high fluorescent yield and which can be excited to fluorescence in the easiest possible manner by means of laser radiation in the longwave visible or NIR spectral range, particularly with light of a helium/neon or diode laser.

The present invention describes marker dyes based on non-symmetrical polymethines which contain a substituted ω-(benz[b]pyran-4-ylidene)alk-1-enyl) unit of general formula (I),

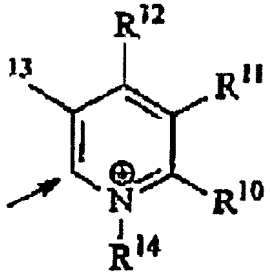


I

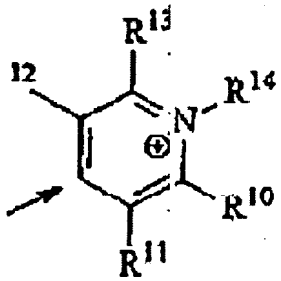
where Z is a substituted derivative of benzooxazol, benzothiazol, 2,3,3-trimethylindolenine, 2,3,3-trimethyl-4,5-benzo3*H*-indolenine, 3- and 4-picoline, lepidine, chinaldine and 9-methylacridine derivatives with the general formulae IIa or IIb or IIc



IIa



IIb



IIc

and where

- X stands for an element of the group O, S, Se or the structural element N-alkyl or $C(alkyl)_2$,
- N stands for the numerical value 1, 2 or 3,
- R^1-R^{14} are equal or different and can be hydrogen, one or more alkyl, aryl, heteroaryl or heterocycloalipathic fragments, a hydroxy or alkoxy group, an alkylsubstituted or cyclical amine function and/or two fragments in ortho position to each other, for example R^{10} and R^{11}, can together form another aromatic ring,
- At least one of the substituents R^1-R^{14} can be a solubilizing or ionizable or ionized substituent, like cyclodextrine, sugar, SO_3^-, PO_3^{2-}, COO^-, or NR_3^+, which determines the hydrophilic properties of these dyes; here it is possible that this substituent can be bound to the marker dye by means of a spacer group,
- At least one of the substituents R^1-R^{14} can stand for a reactive group which facilitates a covalent linking of the dye to the aforementioned carrier molecules, while this substituent can also be bound to the dye by means of a spacer group, and
- R^1 is a substituent which has a quarternary C-atom in alpha-position relative to the pyran ring. Examples for such substituents are t-butyl (-$C(CH_3)_3$) and adamantyl (-$C_{10}H_{15}$/tricyclo[3.3.1.$1^{3,7}$]decyl).

Subclaims 2 to 20 list specific embodiment forms and applications of the marker dyes.

These substituted derivatives of indol, heteroindol, pyridine, chinoline or acridine of the general formula I can be used as dyes for the optical marking of organic or inorganic microparticles, for example of proteins, nucleic acids, DNA, sugars, biological cells, lipids, drugs or organic or inorganic polymeric carrier substances.

Here, the marking of particles can be done by the formation of ionic interaction between the markers of general formula I and the substances to be marked.

The functional groups of these markers activated with regards to nucleophiles can couple covalently with an OH, NH_2 or SH function, which

therefore creates a system for the qualitative and quantitative determination of organic and inorganic substances, like said proteins, nucleic acids, DNA, sugars, biological cells, lipids, drugs or organic or inorganic polymers.

The coupling reaction can take place in an aqueous or mostly aqueous solution, preferably at room temperature. During this a conjugate with fluorescent properties is created.

By means of the preparation of non-symmetrical polymethines, which on the one hand have an easily derivatizable heterocycle of the type of the pyridine, chinoline, indol, heteroindol or acridine derivatives and on the other hand have a novel 6-ring heterocycle, in particular the following advantages are achieved:

Trimethines already absorb in the spectral range >650nm and have a significantly improved photochemical and thermal stability when compared with polymethines known so far in the art which have absorption maxima >650nm (penta- and heptamethines).

By means of molecular engineering, it is possible to control the position and intensity of the absorption and emission maxima at will and to adapt them to emission wavelengths of different excitation lasers, in particular NIR laser diodes.

The marker dyes can be produced by a relatively simple two-stage synthesis with which a variety of dyes with functionalities that differ, for example, with regards to the total charge of the dye and the number, specificity and reactivity of the activated group used for the immobilization can be provided in a manner that is specific to the respective application.

Compounds with the general formula I as well as systems derived from them (conjugates) can be used in optical, in particular fluorescence optical qualitative and quantitative determination methods for the diagnosis of cell properties, in biosensors (point-of-care measurements), exploration of the genome and in miniaturization technology. Typical applications lie in the fields of cytometry, cell sorting, fluorescence correlation spectroscopy (FCS), ultra-high throughput screening (UHTS), multicolor fluorescence in situ hybridization (FISH) and in microarrays (gene and protein chips).

Here, a microarray is a grid-like arrangement of molecules immobilized on at least one surface which can be used for the study of the interaction between receptors and ligands. A grid-like arrangement means more than two molecules which are different from each other and which are immobilized in different, predefined regions of known positions on a surface.

A receptor is a molecule which has an affinity to a given ligand. Receptors can be naturally occurring or artificially produced molecules.

Receptors can be used in their pure state or bound to other species. Receptors can be bound covalently or non-covalently either directly or via certain coupling mediators to a bonding partner.

Examples of receptors which can be detected by means of this invention include agonists and antagonists for cell membrane receptors, toxins and other poisonous substances, viral epitopes, hormones like opiates and steroids, hormone receptors, peptides, enzymes, enzyme substrates, active substances that act as co-factors, lectines, sugars, oligonucleotides, nucleic acids, oligosaccharides, cells, cell fragments, tissue fragments, proteins and antibodies, but they are not limited to the named substances.

A ligand is a molecule that is recognized by a particular receptor. Examples of ligands which can be detected by this invention include agonists and antagonists for cell membrane receptors, toxins and other poisonous substances, viral epitopes, hormones like opiates and steroids, hormone receptors, peptides, enzymes, enzyme substrates, active substances that act as co-factors, lectines, sugars, oligonucleotides, nucleic acids, oligosaccharides, cells, cell fragments, tissue fragments, proteins and antibodies, but they are not limited to the named substances.

The invention is subsequently to be illustrated in more detail by means of embodiment examples and the drawings.

In the accompanying drawings,

Figure 1 shows the structural formula of benzopyrylium salt 2a,

Figure 2 shows the synthesis and structural formula of benzopyrylium salt 2b,

Figure 3 shows the synthesis and structural formula of trimethine OB11 (DY-630),

Figure 4 shows the fluorescent spectra of OB15 (DY-635) in an aqueous solution and bound to bovine serum albumin (BSA),

Figure 5 shows the fluorescent excitation spectra of OB15 (DY-635) in an aqueous solution and bound to bovine serum albumin (BSA).

Embodiment Examples:

1. Instruction for the preparation of 11-(2,2-dimethylethyl)-9-methyl-1H,2H,3H,5H,6H,7H-pyrano[2,3-f]pyrido[3,2,1-ij]chinolin-12-ium tetrafluoroborate 2b (BS28), cf. Figure 2:

50ml of a 1.0 molar solution of methylmagnesiumbromide in dibutylether are added drop by drop to a cooled solution of 7.3g (0.0245mol) 11-(2,2-dimethylethyl)-1H,2H,3H,5H,6H,7H-pyrano[2,3-f] pyrido[3,2,1-ij]chinolin-9-on in 50ml ethylenglycol-dimethylether. The mixture was heated to a temperature of 40 degrees C for a time span of 30 minutes. After cooling down to 0 degrees C, 70ml of a saturated NH_4Cl solution and diluted hydrochloric acid were added for hydrolysis. The organic phase was separated and extracted using 4 x 10ml diethylether. The solvent was removed in a rotary evaporator and the oily residue was dissolved in 20ml pure acetic acid. The addition of 3ml HBF_4 (48 – 50%) and the dilution with diethylether created a precipitant which is filtered out and recrystallized from pure acetic acid.

A yield of 3.35g (35%), melting point 175 – 180 degrees C. – ^1H NMR (400MHz, $CDCl_3 + CF_3CO_2D$): 1.43 (s, 9H), 1.90 (m, 2H), 2.06 (m, 2H), 2.67 (m, 2H), 2.92 (m, 2H), 3.35 (m, 2H), 3.57 (m, 2H), 3.95 (s, 3H), 6.90 (s, 1H), 7.58 (s, 1H): $-C_{20}H_{26}BF_4NO$ (383.24): calculated C 62.68, H 6.84, N 3.65, found C 63.06, H 6.72, N 3.48.

2. General instruction for the preparation of the non-symmetrical trimethines OB 11, OB 14, OB 15 and OB 20:

0.01mol of the corresponding 4-methyl-benzopyrylium-tetrafluoroboratee according to formula 2a (BS4) or 2b (BS28) (cf. Figure 1 and 2) and 0.01mol methylene-active N-heterocycle were dissolved in 20ml acetanhydride and after the addition of 2.0g of triethoxymethane and 5ml pyridine heated for about

10 minutes. The crude dye product was precipitated with 30ml of diethylether after the solution had cooled down to room temperature. The precipitate was filtered out and purified by means of column chromatography.

3. 1-(5-carboxypentyl)-3,3-dimethyl-2-[3-(7-N,N-diethylamino-2-(1,1-dimethylethyl)-4H-benzopyran-4-ylidene)-1-propenyl]-3H-indolium-5-sulfonate OB 11 (DY-630):

0.01mol of 2a and 0.01 mol of 1-(5-carboxypentyl)-2,3,3-trimethyl-3H-indolium-5-sulfonate were transformed according to the general specification 1, see Figure 3. Column chromatography: SiO_2, eluent ethanol. Yield of 3.2g (50%), melting point 280-282 degrees C. – ^1H NMR (400 MHz, DMSO-d6): 1.10 – 1.86 (m, 27H), 2.16 (m, 2H), 3.54 (m, 4H), 4.13 (m, 2H), 6.58 (d, 1H), 6.74 (s, 1H), 6.97 (s, 1H), 7.06 (d, 1H), 7.14 (d, 1H), 7.36 (d, 1H), 7.68 (d, 1H), 7.78 (s, 1H), 8.08 (d, 1H), 8.32 (t, 1H) – ^{13}C NMR (100 MHz, DMSO-d6): 12.30, 21.51, 24.32, 25.66, 26.56, 27.55, 34.07, 36.37, 43.72, 44.22, 48.87, 96,36, 99.40, 104.11, 109.85, 110.28, 112.48, 113.27, 119.66, 126.09, 140.23, 141.81, 145.59, 147.09, 162.14, 172.33, 174.64 – MS (FAB in dmba): 657 ($M + Na^+$), 635 ($M + H^+$), 391, 359, 258, 257 – $C_{36}H_{46}N_2O_6S$ (634.83): calculated C 68.11, H 7.30, N 4.41, found C 68.25, H7.33, N 4.39.

4. 1-(3-hydroxypropyl)-4-[3-(7-N,N-diethylamino-2-(1,1-dimethylethyl)-4H-benzopyran-4-ylidene)-1-propenyl]-chinolinium-tetrafluoroborate OB 14:

0.01mol of 2a and 0.01mol of 1-(3-hydroxypopyl)-4-methylchinolinium-iodide were transformed according to general specification 1. Column chromatography: SiO_2, eluent toluol/ethanol 1/1. Yield of 2.4g (42%), melting point 162-164 degrees C. – ^1H NMR (400 MHz, $CDCl_3$): 1.17 (t, 6H), 1.32 (s, 9H), 2.14 (m, 2H), 2.25 (s, 1H), 3.39 (q, 4H), 3.71 (m, 2H), 4.89 (m, 2H), 6.31 (d, 1H), 6.56 (s, 1H), 6.62 (m, 2H), 7.01 (d, 1H), 7.60 (t, 1H), 7.67 (d, 1H), 7.77 (d, 1H), 7.84 (t, 1H), 7.93 (d, 1H), 8.12 (t, 1H), 8.31 (d, 1H), 9.27 (d, 1H). –^{13}C NMR (100 MHz, $CDCl_3$): 12.52, 28.08, 32.01, 36.20, 44.61, 52.53, 57.52, 97.05, 97.94, 109.58, 109.94, 110.91, 111.77, 113.61, 117.72, 124.79, 125.38, 125.50, 127.13,

133.64, 137.96, 140.92, 142.20, 144.96, 150.87, 151.74, 155.40, 167.12 – MS (FAB in dmba): 483 (M^+) –$C_{32}H_{39}BF_4N_2O_2$ (570.48): calculated C 67.37, H 6.89, N4.91, found C 67.30, H 6.92, N 4.89.

5. 1-(5-carboxypentyl)-3,3-dimethyl-2-[3-(11-(2,2-dimethylethyl)-1H,2H,3H,5H,6H,7H-pyrano[2,3-f]pyrido[3,2,1-ij]chinoline-9-ylidene)-1-propenyl]-3H-indolium-5-sulfonate OB 15 (DY-635):

0.01mol of 2b and 0.01 mol of 1-(5-carboxypentyl)-2,3,3-trimethyl-3H-indolium-5-sulfonate were transformed according to the general specification 1.

Column chromatography: SiO_2, eluent ethanol. Yield of 2.9g (44%), melting point >300 degrees C. – ^1H NMR (250 MHz, DMSO-d6): 1.10 – 1.56 (m, 19H), 1.91 (m, 4H), 2.08 (m, 4H), 2.83 (m, 4H), 3.38 (m, 4H), 4.03 (m, 2H), 6.45 (d, 1H), 6.97 (s, 1H), 7.13 (d, 1H), 7.26 (d, 1H), 7.62 (d, 1H), 7.73 (s, 1H), 7.78 (s, 1H), 8.23 (t, 1H) – ^{13}C NMR (62 MHz, DMSO-d6): 19.40, 20.43, 24.86, 25.98, 26.61, 27.16, 27.76, 27.85, 28.94, 35.17, 36.71, 43.40, 48.45, 49.04, 49.63, 99.24, 102.90, 105.09, 109.69, 110.03, 112.96, 119.71, 121.85, 123.50, 139.89, 142.18, 144.84, 145.76, 148.56, 148.86, 151.59, 170.08, 171.37 – MS (ESI): 681 ($M + Na^+$), 659 ($M + H^+$), 352 – $C_{38}H_{46}N_2O_6S$ (658.12): calculated C 69.27, H 7.34, N 4.25, found C 69.20, H 7.37, N 4.29.

6. 1-(5-carboxypentyl)-4-[3-(7-N,N-diethylamino-2-(1,1-dimethylethyl)-4H-benzo-pyran-4-ylidene)-1-propenyl]-chinolinium-6-sulfonate OB 20:

0.01mol of 2a and 0.01mol 1-(5-carboxypentyl)-4-methyl-chinolinium-6-sulfonate were transformed according to general specification 1.

Column chromatography: SiO_2, eluent ethanol. Yield of 2.1g (35%), melting point >300 degrees C. – $C_{35}H_{42}N_2O_6S$ (618.76): calculated C 67.93, H 6.84, N 4.53, found C 67.73, H 6.93, N 4.29.

7. Preparation of the NHS ester of OB 11 (DY-630) with N-hydroxysuccinimide (NHS)/N,N'-dicylco-hexylcarbodiimide (DCC)

15mg OB 11 (DY-630), 14mg DCC and 4mg NHS were dissolved in 1ml dry DMF. After this, 1µl of thriethylamine were added. The reaction mixture was stirred for 24 hours at room temperature and then filtered. The solvent was then drawn off, the residue was washed with ether. This reaction was quantitative.

8. Preparation of the NHS ester of OB 15 (DY-635) with N-hydroxysuccinimide (NHS)/N,N'-dicylco-hexylcarbodiimide (DCC)

The process was analogous to example 7. This reaction also was quantitative.

9. Excitation and emission spectra of 1-(5-carboxypentyl)-3,3-dimethyl-2-[3-(11-(2,2-dimethylethyl)-1H,2H,3H,5H,6H,7H-pyrano[2,3-f]pyrido[3,2,1-ij]chinoline-9-ylidene)-1-propenyl]-3H-indolium-5-sulfonate OB 15 (DY-635)

The diagram in Figure 4 shows the emissions spectra and the diagram Figure 5 shows the excitation spectra of 1-(5-carboxypentyl)-3,3-dimethyl-2-[3-(11-(2,2-dimethylethyl)-1H,2H,3H,5H,6H,7H-pyrano [2,3-f] pyrido[3,2,1-ij]chinolin-9-ylidene)-1-propenyl]-3H-indolium-5-sulfonate when in water and when non-covalently bound to bovine serum albumin (BSA), with the more intense spectrum being the one of the BSA conjugate. The concentration of both dye solutions was identical for these measurements.

10. General specification for the marking of proteins

The protein marking was done in a 50mM bicarbonate buffer (pH 9.0). A parent solution with 0.5mg reactive dye (for example OB 11-NHS-ester, M = 732 g * mol^{-1}) in 100µl DMF was created. The protein, for example avidine (M = 66000 g * mol^{-1}) was dissolved step by step in portions of 1mg in 200ml bicarbonate buffer; after this, varying volumes of the different and – if necessary – diluted dye parent solutions were added to the different protein aliquots. The reaction mixtures were then stirred for one to two hours at room temperature. The free dye was separated from the marked proteins by means of gel chromatography (Sephadex G25 medium, eluent PBS pH 7.2 22mM).